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LIS1 Association With Dynactin is Required for Nuclear Motility and Genomic Union in the Fertilized Mammalian Oocyte

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Mutations in the human *LIS1* gene cause the devastating brain disorder lissencephaly. LIS1 also regulates microtubule dynamics; it interacts with the molecular motor cytoplasmic dynein and its cofactor dynactin, and is necessary for neuronal migration. Recently, LIS1 has been suggested to mediate pronuclear migration during fertilization. Here we use rhesus monkey and bovine oocytes, as well as pronucleate-stage bovine zygotes, to determine: Lis1 RNA expression using reverse transcription-polymerase chain reaction; LIS1 protein association with dynactin using immunoprecipitation, Western blot analysis, and immunocytochemistry; and LIS1 function in mediating genomic union using antibody transfection. We find that Lis1 RNA expression increases during fertilization, that LIS1 and dynactin subunit p150^{Glued} co-immunoprecipitate and co-localize to pronuclear surfaces, and that anti-LIS1 antibodies transfected into zygotes dramatically inhibit pronuclear migration and apposition. LIS1 is, therefore, essential to mediate genomic union in a process that involves the dynein-dynactin complex. These results shed light on an additional role for LIS1 and raise implications for human reproduction. Cell Motil. Cytoskeleton 56: 245–251, 2003. © 2003 Wiley-Liss, Inc.

Key words: nuclear movement; microtubules, molecular motor; fertilization; LIS1 inhibition

INTRODUCTION

Human type I lissencephaly is a severe brain disorder caused by the failure of neurons to migrate from the paraventricular zone to the cerebral cortex during development [Dobyns et al., 1993]. This results in disorganized cortical layers and reduced gyri, inducing epilepsy and severe mental retardation with death at an early age. Haplo-insufficiency of the Lis1 gene product leads to both isolated lissencephaly sequence (ILS) and Miller-Dieker Syndrome (MDS), which together comprise a majority of cases seen in the clinic [Lo Nigro et al., 1997]. Whereas total loss of Lis1 is embryonic lethal, heterozygous mutations generated in mice result in neuronal migration defects [Hirotsune et al., 1998]. The LIS1 protein, also a subunit of platelet-activating factor acetylhydrolase

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(PAFAH), has been shown to modulate microtubule dynamics in vitro [Sapir et al., 1997].

LIS1 shares 42% identity with NUDF, one of many nuclear distribution (NUD) proteins in the filamentous fungus *Aspergillus nidulans* [Xiang et al., 1995]. During development in *A. nidulans*, nuclei migrate into the germtube and distribute within the cell, and many of the genes that regulate this process share striking homology to members of the cytoplasmic dynein-dynactin motor complex, including *NUDM*, a homologue of dynactin subunit $p150^{Glued}$ [Xiang and Morris, 1999]. Mutations in *Drosophila LIS1*, *C. elegans LIS1*, and the yeast homologue, *PAC1*, generate dynein-related defects in nuclear migration, nuclear orientation, and oogenesis [Swan et al., 1999; Dawe et al., 2001; Geiser et al., 1997].

Fertilization in most mammals requires a sperm aster, a radial array of microtubules nucleated by the sperm centrosome, upon which the egg-derived female pronucleus migrates to the sperm-derived male pronucleus in a process mediated by the dynein-dynactin motor complex [Payne et al., 2003; Reinsch and Karsenti, 1997; Schatten, 1994]. Surprisingly, rodents do not utilize a sperm aster during fertilization, precluding their use for investigating sperm aster-mediated motility in mammals [Schatten, 1994], though Lis1 expression was recently identified in mouse oocytes and zygotes [Cahana and Reiner, 1999]. Because of the relationship that exists among LIS1, dynein, dynactin, and nuclear migration in non-mammalian species, we questioned whether Lis1 is expressed in rhesus monkey and bovine oocytes, and whether LIS1 is required for pronuclear motility and union in bovine zygotes.

MATERIALS AND METHODS

In Vitro Maturation and In Vitro Fertilization

Rhesus monkey and bovine in vitro maturation, along with bovine in vitro fertilization, were carried out according to standard protocols [Hewitson et al., 1998; Navara et al., 1994]. Rhesus oocytes were obtained from the Assisted Reproductive Technology (ART) core facilities at the Oregon National Primate Research Center and Pittsburgh Development Center, bovine oocytes were obtained from a local abattoir and BOMED, Inc. (Madison, WI), and bull semen was obtained from American Breeders Service (DeForest, WI). Rhesus oocytes were cultured at 37°C under 5% CO_2, and bovine oocytes and sperm were incubated at 39°C under 5% CO_2, for up to 20 h.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA from rhesus and bovine oocytes, as well as from bovine zygotes, was isolated and prepared using the StrataPrep® Total RNA Microprep Kit following the manufacturer's protocol (Stratagene, La Jolla, CA). Reverse transcription (RT) and polymerase chain reaction (PCR) amplification of cDNA were performed on the PTC-200 DNA Engine Cycler from MJ Research, using the ProSTAR™ HF Single-Tube RT-PCR System (High Fidelity) from Stratagene. Oligonucleotide primers designed for Lis1 RT-PCR yield a 329-bp product: 5′-GTCGTAGCAACAAAGGAATGC-3′ and 5′-CGCTTGTTCTTGTAATCCCATAC-3′ (primers LIS1(71)1001F and LIS1(71)1329R, respectively) [Lo Nigro et al., 1997]. For β-actin RT-PCR, primers yield a 225-bp product: 5′-CTGGCATTGTCATGGACTCT-3′ and 5′-TCGAAGTCTAGGGCGACATA-3′. Control primers provided by the ProSTAR™ system were used to amplify control products with a size of 500 bp. This control amplification was recommended by the manufacturer to ensure that the kit reagents were working properly. The reverse transcription and PCR steps occurred consecutively in the same tube: reverse transcription was performed at 42°C for 15 min and PCR amplification was carried out with an initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. A final extension of the PCR products occurred at 68°C for 10 min. A negative control reaction was carried out excluding the reverse transcriptase from the reaction mixture. RT-PCR fragments were electrophoresed on 2.5% (w/v) agarose gels and referenced with the 100-bp ladder by Life Technologies. Visualization and analysis of RT-PCR products was achieved using the Gel Doc 2000™ system from Bio-Rad (Richmond, CA). Quantitation of RT-PCR was performed by using the software included with the Gel Doc Imaging system, comparing levels of the Lis1 products with the normalized standards of the β-actin products.

DNA Sequencing and Sequence Alignment

PCR products were end-sequenced using the Lis1 oligonucleotide primers with dye terminators (373 DNA ABI Sequencer; Perkin-Elmer Biosystems). The cDNA sequences for the rhesus and two bovine Lis1 fragments were submitted to GenBank and given the accession numbers AY260741, AY260742, and AY260743, respectively. Sequence alignments were performed using ClustalW Multiple Sequence Alignment computer program, comparing the bovine and rhesus cDNA sequences to human *LIS1* exons 8, 9, and 10 (GenBank accession numbers U72339, U72340, and U72341, respectively) [Lo Nigro et al., 1997]. Human *LIS1* coding sequence used in the alignment encompasses bases 534–650 from U72339, bases 295–396 from U72340, and bases 404–513 from U72341. Deduced protein sequence was generated from the bovine and rhesus nucleotide sequences.

Immunoprecipitation and Western Blot Analysis

SDS-PAGE and Western Blotting of bovine oocytes and zygotes were performed following standard protocols [Harlow and Lane, 1988], and immunoprecipitations of $p150^{Glued}$ dynactin were carried out using Protein A Sepharose 4 Fast Flow medium according to the manufacturer's recommendations (AP Biotech). A concentration of 2.5 μg/ml anti-$p15^{Glued}$ antibody was used for the immunoprecipitation reactions. Mature bovine oocytes were either lysed immediately or fertilized in vitro and cultured until 16 h post-insemination, at which time they were lysed and prepared for antibody incubations. Protein concentrations were determined by the Bradford assay, and equal amounts of protein were added to each lane of polyacrylamide gels. Samples were loaded onto 4–20% gradient Tris-HCl gels, and processed by SDS-PAGE and Western Blotting onto PVDF membranes. Gels were silver stained to verify the presence and relative amounts of protein. LIS1 was identified on the Western blots using goat and rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1.25 μg/ml. Monoclonal anti-dynactin $p15^{Glued}$ antibody (BD Biosciences) was also used at 1.25 μg/ml. HRP-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR) and Jackson ImmunoResearch (West Grove, PA) and used at a 1:5,000 dilution. Protein bands were visualized using the ECL Plus system and HyperFilm (AP Biotech), referenced to Kaleidoscope pre-stained standards (Bio-Rad), and analyzed by a densitometer.

Immunocytochemistry and Antibody Transfection

All zygotes were processed for immunocytochemistry (ICC) using previously described methods [Navara et al., 1994; Payne and Schatten, 2003], with fixation in 2% formaldehyde for 40 min at room temperature, followed by permeabilization with 0.1% TX-100 in 10 mM PBS for an additional 40 min. After blocking with 0.3% BSA in 10 mM PBS, samples were incubated with primary antibodies. LIS1 was identified by ICC using goat and rabbit polyclonal antibodies (Santa Cruz Biotechnology) at a concentration of 12.5 μg/ml. Monoclonal anti-dynactin $p150^{Glued}$ antibody (BD Biosciences) was also used at 12.5 μg/ml. Control experiments were performed by pre-absorbing the anti-LIS1 antibodies with LIS1 blocking peptide (Santa Cruz Biotechnology), by pre-incubating the anti-dynactin $p150^{Glued}$ antibodies with human endothelial cell lysates provided by the Pittsburgh Development Center, and by using pre-immune mouse IgG antibodies (Chemicon, Temecula, CA) at 12.5 μg/ml. Following washes, secondary antibodies were then applied, using AlexaFluor-conjugated antibodies (Molecular Probes) at a 1:200 dilution. Samples were then stained with 10 μg/ml TOTO-3 (Molecular Probes) to label the DNA. Fixed zygotes were imaged using a Leica TCS SP2 spectral confocal microscope at laser lines 488, 568, and 633 nm.

Antibody transfection of bovine zygotes at 12 h post-insemination was achieved using the Chariot™ reagent following the manufacturer's protocol (Active Motif). Briefly, for each antibody transfection into 20 zygotes, a 1:5 dilution of anti-LIS1 antibodies, or a 1:20 dilution of pre-immune IgG antibodies, was mixed with a 1:10 dilution of Chariot™ reagent in a total volume of 20 μl. This mixture was then incubated at room temperature for 30 min. Following this antibody-Chariot™ binding step, the entire 20 μl volume was added to one well of a 96-well plate containing 20 zygotes in 80 μl of protein-free TL-Hepes medium. The samples were incubated at 39°C for 1 h, after which an additional 100 μl of protein-containing TL-Hepes medium was added to the well; samples were cultured for an additional 7 h, and fixed for immunocytochemistry at 20 h post-insemination. For these transfection experiments, antibodies were dialyzed overnight using Slide-A-Lyzer cassettes (Pierce, Rockford, IL) in multiple changes of 10 mM PBS to remove sodium azide from the storage buffer.

RESULTS

Cloning and Expression of Lis1 in Rhesus Monkey and Bovine Oocytes and Zygotes

To characterize the expression of Lis1 in mammalian oocytes that utilize a sperm aster during fertilization, we isolated total RNA from rhesus monkey and bovine oocytes and performed RT-PCR. Amplification of cDNA was performed using oligonucleotide primers that recognize a coding region within the human *LIS1* gene and that were previously used to predict outcome associated with ILS and MDS [Lo Nigro et al., 1997]. This portion of the gene is relevant to the current study, as it is a region that might show genetic variability across species. RT-PCR using these primers yielded 329-bp products (Fig. 1A). Two bovine Lis1 products (1031A and 1031B) and one rhesus Lis1 product (Rh6) were then sequenced and compared to the human *LIS1* coding sequence of that region (Fig. 1B). Human Lis1 is 95% identical to rhesus Lis1 and 91% identical to bovine Lis1 over the length of the isolated cDNA. This high degree of homology is not entirely surprising, since the recent comparison between human and rhesus UDP-glucuronosyl-transferase also revealed 95% identity, reflecting the extensive conservation of sequence among primates [Dean et al., 2002]. The deduced protein sequence is shown in Figure 1C.

Mammalian Lis1 expression at fertilization was examined further by performing RT-PCR and comparing

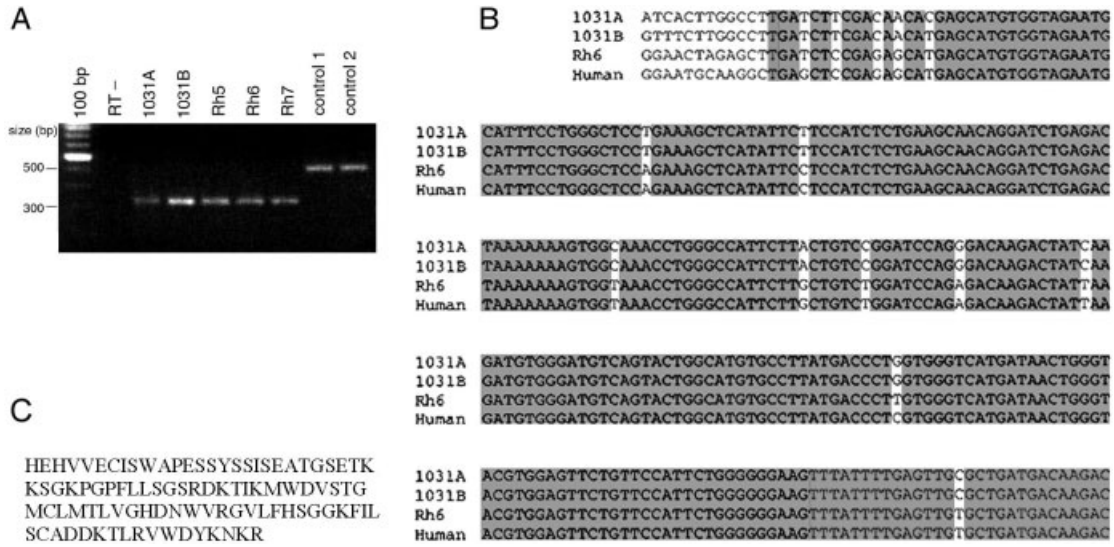

Fig. 1. RT-PCR and cDNA sequence analysis of bovine and rhesus monkey Lis1. **A:** Detection of the 329-bp Lis1 product, reverse transcribed and amplified from total RNA, isolated from bovine (1031A, 1031B) and rhesus (Rh5, Rh6, and Rh7) oocytes. Positive controls 1 and 2 yield a 500-bp product, and negative control (RT−) shows no product. **B:** Sequence comparison among bovine (1031A, 1031B), rhesus (Rh6), and human Lis1 cDNA. Data are taken from GenBank as listed in Materials and Methods, and sequence analysis was performed with the Multiple Sequence Alignment computer program using the ClustalW algorithm. Conserved residues are highlighted with *gray boxes*. **C:** The deduced protein sequence of this portion of the Lis1 gene, generated from the bovine and rhesus nucleotide sequences.

the levels of Lis1 products isolated from unfertilized bovine oocytes and zygotes against the levels of β-actin products (Fig. 2A). Semi-quantitation of the Lis1 products indicates that levels in the zygotes are nearly double those in the oocytes (~190%). These results suggest that Lis1 mRNA might be transcribed in the fertilized zygote. Recent evidence has shown that 1-cell bovine embryos are indeed transcriptionally and translationally active for genes important for embryonic development [Memili and First, 1999]. Lis1 transcription has also been reported to occur in mouse zygotes, indicating the importance of this gene's expression in the early mammalian embryo [Cahana and Reiner, 1999].

LIS1 Associates With Dynactin in Pronucleate-Stage Bovine Zygotes

Western blot analysis was performed to determine whether LIS1 translation occurs during fertilization. LIS1 protein is detected here as a single 45-kD band in unfertilized bovine oocytes and zygotes (Fig. 2B), with densitometry analysis showing levels approximately one-third (~30%) higher in zygotes than in oocytes. This putative increase in mRNA and protein expression in the zygote suggests a potential function for LIS1 during fertilization and early embryogenesis.

LIS1 interacts with the p150^{Glued} subunit of dynactin in embryonic, neonatal, and adult mammalian neurons, and its association with dynactin is particularly enriched at the cortical plate and marginal zone of the embryonic brain,

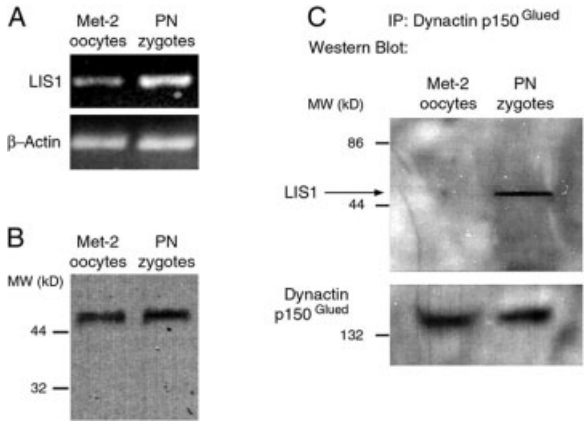

Fig. 2. LIS1 RNA expression, protein expression and co-immunoprecipitation with dynactin. **A:** RNA from 5 meiotically arrested (Met-2) bovine oocytes or 5 pronucleate (PN) bovine zygotes was isolated, reverse transcribed, and amplified for each RT-PCR reaction. Lis1 and control β-actin products were electrophoresed, imaged, and quantified. Semi-quantitative comparison between the levels of Lis1 products in oocytes and zygotes and the normalized standards of β-actin products suggests that Lis1 transcription may occur upon fertilization. **B:** Protein from bovine Met-2 oocytes and PN zygotes was extracted, resolved by SDS-PAGE, and subjected to Western blot analysis using anti-LIS1 antibodies. Single 45-kD bands are detected in both oocytes and zygotes. **C:** SDS-PAGE and Western blot analysis following immunoprecipitation (IP) of dynactin p150^{Glued} from bovine Met-2 oocytes and PN zygotes. LIS1 is enriched in the dynactin immunoprecipitate prepared from zygotes, but not from oocytes. Dynactin p150^{Glued} is detected in both samples.

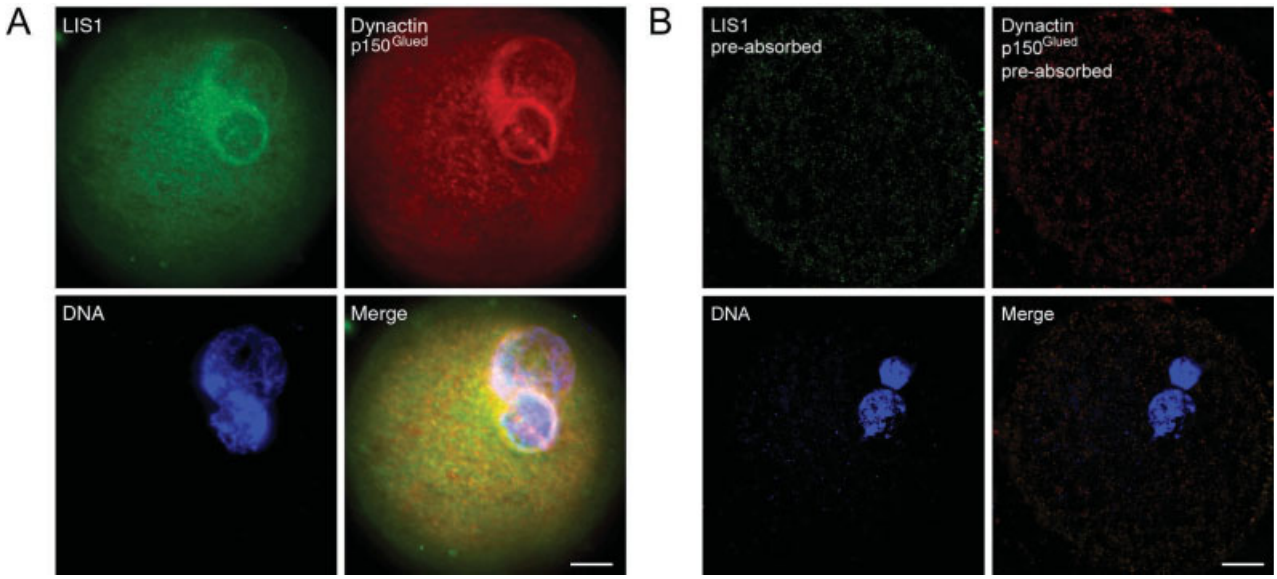

Fig. 3. LIS1 and dynactin co-localize around the pronuclear surfaces in zygotes. **A:** Pronucleate-stage bovine zygote shows both LIS1 (*green*) and Dynactin p150^{Glued} (*red*) concentrated around the two pronuclei (DNA; *blue*). Co-localization is detected in the three-channel overlay (Merge). **B:** Pre-absorption of anti-LIS1 antibodies with their antigens and pre-incubation of anti-p150^{Glued} antibodies with human endothelial cell lysates result in a loss of LIS1 and dynactin detection in zygotes. Scale bar = 10 μm.

sites of neuronal migration [Smith et al., 2000]. We, therefore, questioned whether LIS1 associates with dynactin in bovine zygotes during pronuclear migration. Co-immunoprecipitation experiments show that the anti-dynactin p150^{Glued} antibody pulls down LIS1 from pronucleate-stage zygotes, but not from unfertilized oocytes (Fig. 2C). The p150^{Glued} subunit is enriched in both samples. This result indicates that LIS1 interacts with dynactin at fertilization, perhaps to mediate pronuclear motility.

Co-Distribution of LIS1 and Dynactin to Pronuclear Surfaces

The distribution of LIS1 and dynactin p150^{Glued} during pronuclear migration and apposition was then characterized by confocal microscopy. Both proteins localize at the surfaces of the two pronuclei (Fig. 3A). While some dim cytoplasmic foci can be seen for LIS1 and dynactin, both proteins concentrate along the pronuclear rims. Pre-absorption of anti-LIS1 antibodies with their antigens and pre-

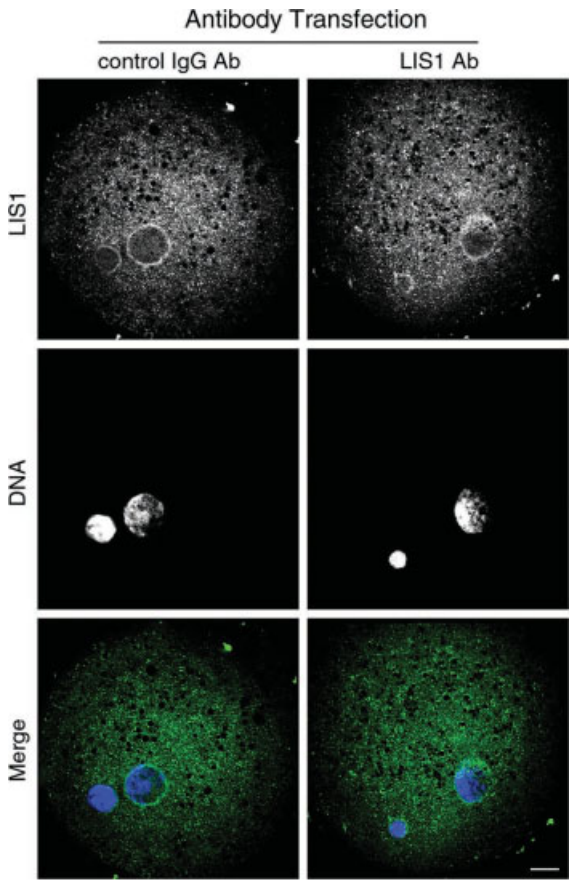

Fig. 4. LIS1 is required for pronuclear apposition. **Left:** Normal inter-pronuclear distances are observed when control IgG antibodies are transfected into bovine zygotes. LIS1 concentrates around the two pronuclei, with additional punctate cytoplasmic foci. **Right:** Transfection using anti-LIS1 antibodies inhibits pronuclear apposition, while the distribution of LIS1 resembles control conditions. Scale bar = 10 μm.

TABLE I. Anti-LIS1 Antibodies Inhibit Pronuclear Union During Fertilization

Transfection of zygotes at 12 h with Chariot™ reagent + antibodies:	Distance between pronuclei at 20 h	
	≥10 μm apart	<10 μm apart
Pre-immune mouse IgG	0% (0/92)	100% (92/92)
Anti-LIS1	81% (69/85)	19% (16/85)

incubation of anti-p150^{Glued} antibodies with human endothelial cell lysates result in no LIS1 or dynactin staining within the zygotes (Fig. 3B). LIS1 has recently been identified to localize at nuclear envelopes in prophase somatic cells [Coquelle et al., 2002], and dynactin has been proposed to interact with cytoplasmic dynein to facilitate nuclear envelope breakdown [Salina et al., 2002]. Another possible function of LIS1 and the dynein-dynactin complex might be to facilitate pronuclear migration.

Requirement of LIS1 for Genomic Union

Co-localization of LIS1 and dynactin to pronuclear surfaces suggests their importance in mediating genomic union. To determine whether LIS1 is required for pronuclear migration and apposition, we transfected antibodies against LIS1 into pronucleate-stage bovine zygotes using Chariot™ reagent [Morris et al., 2001]. Antibody transfection occurred after the formation but before the union of the pronuclei, specifically targeting pronuclear motility. Development of the transfected zygotes was allowed until just prior to mitosis, whereupon distances between the two pronuclei were measured to score the inhibition of genomic union. Measured from surface-to-surface, inter-pronuclear distances ≥ 10 μm reflect inhibition, with 10 μm representing the average pronuclear diameter.

The majority of zygotes transfected with anti-LIS1 antibodies show pronuclei ≥ 10 μm apart (81%; Table I). LIS1 concentrates around both pronuclei and distributes throughout the cytoplasm as punctate foci (Fig. 4). All of the zygotes transfected with pre-immune mouse IgG antibodies display inter-pronuclear distances < 10 μm and normal LIS1 distribution (Table I, Fig. 4). We conclude from these data that LIS1 is essential for pronuclear migration and genomic union. Recent observations of *C. elegans* zygotes noted that pronuclear apposition does not occur when the animals are subjected to Lis1 RNAi [Dawe et al., 2001]. Our results here demonstrate a role for LIS1 on nuclear motility during mammalian fertilization, with implications for human infertility.

DISCUSSION

LIS1 is required for early mouse embryogenesis, with homozygous null mutants exhibiting post-implantation lethality [Hirotsune et al., 1998]. Morphological analysis of homozygous null blastocysts revealed defects in inner cell mass growth and development, resulting in embryonic death prior to neuronal differentiation or migration. While the precise cause of such defects has not yet been identified, the ability of these embryos to develop until implantation suggests that maternally-inherited Lis1 transcripts and protein levels are sufficient in the zygote to permit development in the absence of gene expression. This current study shows that when LIS1 protein is inhibited by antibody transfection into the zygote, specific defects in genomic union are observed.

Co-immunoprecipitation and co-localization of LIS1 with dynactin in the zygote identify a possible role for LIS1 in modulating the dynein-dynactin complex. Recent studies report that LIS1, dynein, and dynactin localize to mitotic kinetochores and microtubule "plus" ends in somatic cells [Faulkner et al., 2000; Coquelle et al., 2002], and that LIS1 interacts with specific regions in the dynein and dynactin molecules [Tai et al., 2002]. Indeed, in unfertilized oocytes LIS1 localizes to regions along the meiotic spindle that are enriched with dynein and dynactin (C. Payne, unpublished observations). Given the proposed role of dynein and dynactin in mediating pronuclear migration and apposition during fertilization [Payne et al., 2003; Reinsch and Karsenti, 1997; Schatten, 1994], LIS1 likely regulates the motor complex at the surfaces of the pronuclei to facilitate nuclear motility and union. Recent evidence shows that when anti-dynein and anti-dynactin antibodies are transfected into pronucleate-stage zygotes, the migration of the female pronucleus is dramatically inhibited [Payne et al., 2003]. Thus, the similarity in outcome of blocking dynein, dynactin, and LIS1 during fertilization suggests their involvement in an interactive complex that mediates pronuclear movement.

It is remarkable that a gene highly expressed in neurons and critical for neuronal migration should also be expressed and functionally important at fertilization. Recent studies illustrate, however, that neurons and gametes share more commonalities than were once appreciated. The RNA binding protein CPEB (cytoplasmic polyadenylation element binding protein), for example, regulates key functions in both cell types [Richter, 2001], and SPNR (spermatid perinuclear RNA-binding protein) shows abundant expression in brain, ovary, and testis [Pires-daSilva et al., 2001]. Thus, proteins essential for proper development of the nervous system might also, if defective, contribute to infertility. With a role identified for LIS1 in mediating pronuclear migration and genomic union, we might now add fertilization defects together with lissencephaly as devastating consequences induced, respectively, by faulty LIS1 protein and gene expression.

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